SUPPORTING INFORMATION

SUPPLEMENTARY METHODS

Selection criteria

The cohort comprised 21 males and 12 females with a median age 63 years (range 23–83 years). For each patient, we obtained neuroimaging data including preoperative tumor volume (contrast enhancement plus central necrosis) and necrotic volume (as potential surrogate of ‘‘perinecrotic’’ tumor hypoxia) from pre-operative contrast-enhanced computer tomography (CT) or magnetic resonance imaging (MRI) scans.[1] Pre-operative CT or MRI scans were imported into the radiotherapy planning system Oncentra Masterplan (Nucletron BV, Veenendal, the Netherlands). The outer rim of contrast enhancing tumor was contoured as a 3-dimensional structure on CT or T1-weighted MRI and the whole volume contained within this structure was defined as tumor volume. The hypodense or T1-hypointense subvolume within the tumor volume was equally contoured and defined as necrotic volume. To verify the comparability of CT-derived and MRI-derived tumor volumes, these volumes were calculated in a sample of cohort patients (n=4) having both preoperative contrast-enhanced CT and contrast-enhanced MRI. In all four cases, the volumes from both sources differed by less than 15%. Furthermore, for each patient of the cohort, tumor volumes were measured by the same operator, independently from the adopted radiological investigation. Thus, we considered the tumor volume calculated from CT and MRI scans as comparable and reliable. Median preoperative tumor volumes were 61.25 (IQR 25.30-107.85) and 13.45 (IQR 1.90-28.68) in patients with glioblastoma and other tumors, respectively.

Histological analysis revealed the following diagnoses: glioblastoma (n = 21), anaplastic astrocytoma (n = 9) and anaplastic oligodendroglioma (n = 3).[1,2] Considering the recruitment interval, data on IDH-, MGMT, 1p/19q-co-deletion, MYB and MYBL-status were not available.[3] The tumor diagnosis was made according to the 2007 WHO Classification of Tumors of the Central Nervous System[2] which was adopted during the patient recruitment (2008-2010) as well as in our previous publication on the present cohort.[1] Accordingly, we divided the patients in the following groups: glioblastoma (WHO grade IV, n=21) or other tumors (WHO grade III, n=12).[1]

31 of the 33 patients underwent radiotherapy (mean total dose 54 Gy, total dose ≤ 54 Gy n=11, > 54 Gy n=20). Radiotherapy total doses ranged from 36 to 60 Gy (mean 54 Gy). 19 of the 31 patients were treated with concomitant and adjuvant temozolomide. Of the 19 patients who received chemotherapy, 18 were treated with a total radiation dose ≤54 and one with a total dose >54 Gy.[1] Mean time interval between surgery and begin of radiotherapy and mean duration of radiotherapy were 20.4±8.7 and 33±11.8 days, respectively.

Data concerning tumor recurrence, cause of death as well as data on progression free survival have been not systematically collected and, thus, not included in the present study. Median survival was 7.3 months for patients with glioblastoma and 23 months for patients with other tumors. Mean time interval between t0 and t3 was 102.7±18.3 days. Mean time intervals between t0 and surgery, t1 and surgery, t1 and starting of radiotherapy, t2 and end of radiotherapy and t3 and end of radiotherapy were 2.9±2.8, 19.3±9.0, 2.4±2.1, 1.8±1.7 and 40.1±20.4 days, respectively.

Biomarker analyses

Plasma β-syn and plasma GFAP were measured using an in-house established digital ELISA assay and the Simoa GFAP Discovery kit on a Simoa HD-X analyzer platform (Quanterix, Billerica, Massachusetts, USA), respectively, as described.[4,5] Plasma NfL and NfH quantification was performed with commercially available kits for the ELLA microfluidic system (Bio-Techne, Minneapolis, USA).[6] The coefficients of intra- and inter-assay variability for all measurements were <10% and <15%, respectively.

Statistical analyses

Statistical analyses were carried out with IBM SPSS Statistics V.21 (IBM Inc., Armonk, USA), GraphPad Prism V.7 (GraphPad Software, La Jolla, USA) and R version 4.2.2 (R foundation, Vienna, Austria).

Chi-Square was adopted for comparison between categorical variables. We performed multivariate linear regression models to adjust for tumor volume the differences in blood biomarkers between the diagnostic groups after the transformation of the dependent variable in the natural logarithmic scale.

The cumulative time-dependent probability of death was calculated by the Kaplan–Meier estimate. The time of entry into the analysis was the date of blood collection, and the time of the endpoint was the date of death or the date of the last follow-up information, whichever came first. We performed univariate and multivariate

Cox regression analyses to test the possible associations between survival and continuous values of each biomarker and/or previously described prognostic factors in diffuse gliomas.[7] For the analysis of survival, each biomarker concentration was natural log-transformed to fulfil the normal distribution. We tested by univariate models the contribution of each possible covariate and then added to the multivariate model only those with significant associations. The results are presented as hazard ratios (HRs) and 95% confidence intervals (CIs). The assumption of proportional hazard was assessed by Schoenfeld residuals.

SUPPLEMENTARY RESULTS

Associations between plasma biomarkers and demographic or clinical variables

Age at baseline was significantly associated with plasma GFAP ($r=0.586$, $p<0.001$), NfL ($r=0.579$, $p<0.001$), NfH ($r=0.461$, $p=0.015$) but not with β-syn. However, patients with glioblastoma did not differ in age from subjects with other tumors ($p=0.082$). Sex showed no association with all biomarkers (β-syn: $p=0.549$, GFAP: $p=0.351$, NfL: $p=0.638$, NfH, $p=0.497$). Furthermore, there was no difference in gender distribution between patients with glioblastoma and patients with other tumors ($p=0.632$).

We found significant correlations at t0 between GFAP and NfL ($r=0.813$, $p<0.001$) or NfH ($r=0.452$, $p=0.021$) levels as well as between NfL and NfH ($r=0.593$, $p=0.001$) but not between β-syn and other biomarkers.

Given that most patients with high radiotherapy total dose received also chemotherapy, comparisons between subgroups with and without adjuvant pharmacotherapy disclosed similar results to those concerning subgroups receiving high radiotherapy total dose not (data not shown).

Time course of plasma biomarkers and effect of therapeutic regimens on biomarker levels in patients with glioblastoma

Patients with glioblastoma (WHO Grade IV, n=21) showed an early increase in β-syn (t1-t0: $p=0.013$; not significant after multiple comparison correction), NfL (t1-t0: $p=0.024$) and NfH (t1-t0: $p=0.016$) levels after surgery. Moreover, there was a non-significant decreasing trend at follow-up for all three biomarkers. Conversely, we did not find an increase in GFAP levels after surgery, rather the marker decreased significantly

at long-term follow-up (t3-t1: p=0.024). The sub-analyses on the influence of surgery or radiotherapy regimens on biomarker concentrations failed to reveal any significant association, probably due to the small sample size.

Associations between blood biomarkers and survival

When the entire cohort was considered at baseline (n=28), univariate Cox regression analyses identified the combination of radio- and chemotherapy as a positive prognostic factor among demographical and clinical variables. Age, the presence of glioblastoma, a multifocal disease were negatively associated with survival, whereas sex and type of surgery did not predict survival (**Table S1**). Following the multivariate Cox analyses, only GFAP showed a trend towards an association with survival, after accounting for all covariates (HR: 1.658 (0.980-2.807), p=0.060) (**Tables S2, S3, S4**). In the sub-analysis on glioblastoma patients, none of the biomarkers remained significantly associated with survival (**Table S5**).

Table S1. Univariate COX regression analyses for demographical, clinical and biomarker variables in the entire cohort.

Variable		Univariate COX regression	
		HR (95% CI)	**p value**
Age	Continuous variable	1.087 (1.045-1.131)	<0.001
Sex	Male	Ref	Ref
	Female	0.630 (0.255-1.554)	0.316
Type of tumor	Other tumors	Ref	Ref
	Glioblastoma	6.002 (1.647-21.871)	0.007
Multifocal disease	No	Ref	Ref
	Yes	2.751 (1.047-7.224)	0.040
Type of surgery	complete resection	Ref	Ref
	incomplete resection	2.041 (0.823-5.059)	0.124
Regimens of adjuvant therapy	RT alone	Ref	Ref
	RT+CT	0.207 (0.075-0.566)	0.002
β-syn	Continuous variable	1.298 (0.827-2.036)	0.257
GFAP	Continuous variable	1.629 (1.237-2.143)	<0.001
NfL	Continuous variable	2.573 (1.561-4.239)	<0.001
NfH	Continuous variable	1.652 (1.107-2.465)	0.014

β-syn: beta-synuclein protein, CI: confidence interval, CT: chemotherapy, GFAP: glial fibrillary acidic protein, HR: hazard ratio, NfH: neurofilament heavy chain protein, NfL: neurofilament light chain protein, Ref: reference, RT: radiotherapy

Table S2. Multivariate COX regression analyses for GFAP in the whole cohort accounting for demographical and clinical variables as covariates.

Variable		Multivariate COX regression	
		HR (95% CI)	p value
GFAP	Continuous variable	1.658 (0.980-2.807)	0.060
Age	Continuous variable	1.090 (1.012-1.174)	0.022
Type of tumor	Other tumors	Ref	Ref
	Glioblastoma	1.284 (0.124-13.328)	0.007
Multifocal disease	No	Ref	Ref
	Yes	3.754 (0.995-14.156)	0.051
Regimens of adjuvant therapy	RT alone	Ref	Ref
	RT+CT	0.875 (0.164-4.669)	0.876

We added to the multivariate model only variables with significant associations with survival at univariate analyses. CI: confidence interval, CT: chemotherapy, GFAP: glial fibrillary acidic protein, HR: hazard ratio, Ref: reference, RT: radiotherapy

Table S3. Multivariate COX regression analyses for NfL in the entire cohort accounting for demographical and clinical variable as covariates.

Variable		Multivariate COX regression	
		HR (95% CI)	p value
NfL	Continuous variable	1.980 (0.872-4.498)	0.103
Age	Continuous variable	1.069 (1.004-1.139)	0.037
Type of tumor	Other tumors	Ref	Ref
	Glioblastoma	1.314 (0.225-7.659)	0.762
Multifocal disease	No	Ref	Ref
	Yes	2.668 (0.850-8.317)	0.093
Regimens of adjuvant therapy	RT alone	Ref	Ref
	RT+CT	0.772 (0.132-4.493)	0.773

We added to the multivariate model only variables with significant associations with survival at univariate analyses. CI: confidence interval, CT: chemotherapy, HR: hazard ratio, NfL: neurofilament light chain protein, Ref: reference, RT: radiotherapy

Table S4. Multivariate COX regression analyses for NfH in the entire cohort accounting for demographical and clinical as covariates.

Variable		Multivariate COX regression	
		HR (95% CI)	**p value**
NfH	Continuous variable	1.014 (0.559-1.839)	0.965
Age	Continuous variable	1.071 (0.997-1.150)	0.059
Type of tumor	Other tumors	Ref	Ref
	Glioblastoma	2.862 (0.686-11.942)	0.149
Multifocal disease	No	Ref	Ref
	Yes	2.836 (0.832-9.661)	0.096
Regimens of adjuvant therapy	RT alone	Ref	Ref
	RT+CT	0.553 (0.111-2.752)	0.469

We added to the multivariate model only variables with significant associations with survival at univariate analyses. CI: confidence interval, CT: chemotherapy, HR: hazard ratio, NfH: neurofilament heavy chain protein, Ref: reference, RT: radiotherapy

Table S5. Univariate COX regression analyses for demographical, clinical and biomarker variables in patients with glioblastoma.

Variable		Univariate COX regression	
		HR (95% CI)	p value
Age	Continuous variable	1.059 (1.011-1.110)	0.015
Sex	Male	Ref	Ref
	Female	0.514 (0.169-1.560)	0.240
Multifocal disease	No	Ref	Ref
	Yes	1.418 (0.381-5.272)	0.603
Type of surgery	complete resection	Ref	Ref
	incomplete resection	1.757 (0.601-5.141)	0.303
Adjuvant therapy	No (surgery only)	Ref	Ref
	Yes	0.136 (0.022-0.824)	0.030
Regimens of adjuvant therapy	RT alone	Ref	Ref
	RT+CT	0.257 (0.073-0.908)	0.035
β-syn	Continuous variable	1.564 (1.002-2.439)	0.134
GFAP	Continuous variable	1.330 (0.849-2.083)	0.213
NfL	Continuous variable	1.768 (0.858-3.641)	0.122
NfH	Continuous variable	1.067 (0.696-1.637)	0.766

β-syn: beta-synuclein protein, CI: confidence interval, CT: chemotherapy, GFAP: glial fibrillary acidic protein, HR: hazard ratio, NfH: neurofilament heavy chain protein, NfL: neurofilament light chain protein, Ref: reference, RT: radiotherapy

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